Archives of Oral Biology 90 (2018) 100–107

Contents lists available at ScienceDirect

Archives of Oral Biology

journal homepage: www.elsevier.com/locate/archoralbio

The role of Hedgehog signaling in cementoblast differentiation

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ARTICLE INFO

Keywords:
Cementoblast
Differentiation
Hedgehog
Purmorphamine
Cyclopamine
Bone morphogenetic protein

ABSTRACT

Objective: It has been well known that Hedgehog (Hh) signaling plays an important role in bone development, however, its function in cementogenesis has not yet been reported. This study was intended to elucidate the role of Hh signaling in cementoblast differentiation.

Design: Expression changes of various Hh signaling components and levels of skeletogenic markers (alkaline phosphatase, osteocalcin, osteopontin) and osteogenic transcription factors (RUNX2, Osterix) by Hh signaling modulators during OCCM-30 cementoblast differentiation were determined by quantitative real-time reverse transcriptase polymerase chain reaction. To investigate effects of Hh signaling modulators on the mineralization of cementoblast, alkaline phosphatase and alizarin red S staining were used. Then, the interaction between Hh and BMP signaling during cementoblast differentiation was evaluated using co-treatment of BMP7 and Hh signaling modulators.

Results: We observed the consistent expression of Hh signaling molecules in the OCCM-30, which were up-regulated during cementoblast differentiation. We also found that the treatment of cells with Purmo, an Hh activator, enhanced cementoblast differentiation by increasing the mRNA expression of skeletogenic markers and osteogenic transcription factors, as well as increasing alkaline phosphate activity and mineralization capability. On the contrary, an Hh antagonist, like Cyclo, effectively inhibited cementoblast differentiation. Furthermore, BMP7 promoted cementoblast differentiation through crosstalk with the Hh signaling.

Conclusion: These results suggest that Hh signaling is involved in cementoblast differentiation, and Hh signaling molecules may therefore represent new therapeutic targets in periodontal treatment and regeneration.

1. Introduction

Cementum is a thin, calcified tissue that covers the surface of the root and helps the tooth remain anchored to the alveolar bone. The maintenance of intact cementum is necessary to prevent root resorption. Cementum is one of the key components of the periodontal attachment apparatus, and it is also essential for the functional regeneration of periodontal tissues (Foster, Popowics, Fong, & Somerman, 2007; Saygin, Giannobile, & Somerman, 2000). Although cementum has a similar biochemical composition to bone, it lacks blood vessels and nerve cells, and it has a very limited remodeling ability compared with bone (Bosshardt, 2005). Cementoblasts, located along the root surface, are responsible for the formation of the cementum matrix and its subsequent mineralization. Similar to bone forming osteoblasts, cementoblasts express non-collagenous proteins such as alkaline phosphatase (ALP), RUNX2, bone sialoprotein (BSP), and osteocalcin (OCN) either *in vivo* or *in vitro* (D'Errico et al., 1997; Matthews et al., 2016; Nemoto et al., 2009). It remains controversial, however, whether cementoblasts and osteoblasts are differentiated from a common periodontal precursor cell (Bosshardt, 2005; Cao et al., 2012). The precise molecular mechanisms regulating cementoblast differentiation have also not yet been delineated.

Much has been learned about tooth development at the molecular level over the past 10 years. Fibroblast growth factor (FGF), bone morphogenetic protein (BMP), hedgehog (Hh), wingless protein family (Wnt), notch, and the transcription factors activated by these proteins are known to be key signal transduction factors in the epithelial-mesenchymal reciprocal induction in the developing tooth (Balic & Thesleff, 2015; Jussila & Thesleff, 2012; Zhang, Chen, Song, Liu, & Chen, 2005). Many of the studies looking at the mechanisms of tooth development are focused on the early stages, such as the cap stage (Cobourne & Sharpe, 2010; Thesleff, Vaahtokari, Vainio, & Jowett, 1996). A recent trend, however, has shifted into examining crown formation followed by root development as well as periodontal tissue formation in functional tooth regeneration (Huang et al., 2008; Menicanin, Hynes, Han, Gronthos, & Bartold, 2015; Oshima & Tsuji,

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https://doi.org/10.1016/j.archoralbio.2018.03.006
Received 13 November 2017; Received in revised form 7 March 2018; Accepted 18 March 2018

Table 1
Oligonucleotide sequences of primers.

Gene	Primer Sequence	Size (bp)	GenBank (Accession No.)
GLI1	F 5′-ATCACCTGTTGGGGATGCTG-3′ R 5′-GCTCACTGTTGATGTGGTGC-3′	153	NM_010296.2
GLI2	F 5′-GGGGATAATGAGGCCAGCG-3′ R 5′-CTGGACTGACAAAGCCCGGA-3′	140	NM_001081125.1
GLI3	F 5′-CCTGAGCCACGGGTTTTGAT-3′ R 5′-GGAAATCGGGTCACCCAACA-3′	181	NM_008130.2
PTC	F 5′- ACCGTGTCCTGAGGTGTCT-3′ R 5′- CTGCTGTGCTTCGTATTGCC-3′	199	NM_008957.2
SMO	F 5′- ACTCCAGTGCCACCAGAAG-3′ R 5′- CTCAAGGGGCACACCTCCT-3′	127	NM_176996.4
DHH	F 5′- AGCAACTTGTGCCTCTGCTA-3′ R 5′- TTGCAACGCTCTGTCATCAG-3′	199	NM_007857.5
IHH	F 5′- GGCCATCACTCAGAGGAGTC-3′ R 5′- GCCGAATGCTCAGACTTGACA-3′	179	NM_010544.2
SHH	F 5′- CAACGTAGCCGAGAAGACCC-3′ R 5′- TGTCTTTGCACCTCTGAGTCAT-3	167	NM_009170.3
LUM	F 5′- AGAGAGTAAGGTCACAGAGGACT-3′ R 5′- ATTCTGGTGCACAGTTGGGT-3′	152	NM_008524.2
ALP	F 5′-TATGGTAACGGGCCTGGCTAC-3′ R 5′-TGCTCATGGACGCCGTGAAGCA-3′	187	NM_007431.2
OCN	F 5′-TGAACAGACTCCGGCGCTAC-3′ R 5′-AGGGCAGCACAGGTCCTAA-3′	172	NM_007541.2
OPN	F 5′-GCCGAGGTGATAGCTTGGCT-3′ R 5′-TGATCAGAGGGCATGCTCAG-3′	177	NM_001204201.1
RUNX2	F 5′-CCAGGCAGGTGCTTCAGAACTG-3′ R 5′-ACATGCCGAGGGACATGCCTG-3′	157	NM_001146038.2
OSX	F 5′- CCTCGCTCTCTCCTATTGCAT-3′ R 5′- GTTGAGGAGGTCGGAGCATA-3′	163	NM_130458.3
β-actin	F 5′-GATCTGGCACCACACCTTCT-3′ R 5′-GGGGTGTTGAAGGTCTCAAA-3′	138	NM_007393.3

2014). Compared to studies on BMP and FGF, which have expanded into new areas (Li, Prochazka, Goodwin, & Klein, 2014; Nie, Luukko, & Kettunen, 2006), the studies on Hh signal transduction have been limited to its role during the early stages of tooth development (Chuong, Patel, Lin, Jung, & Widelitz, 2000; Cobourne, Miletich, & Sharpe, 2004).

Hh genes, first identified in *Drosophila melanogaster*, are segment polarity genes that regulate fetal segmentation and morphology formation in fruit flies. Interestingly, the Hh genes are highly conserved in vertebrates (Bejsovec & Wieschaus, 1993; Chuang & McMahon, 1999; Lee, Zhao, & Ingham, 2016). The Hh gene family in the higher vertebrates has three components, sonic hedgehog (SHH), indian hedgehog (IHH), and desert hedge (DHH), which are not only involved in the formation of the skeletal system, but also function as signaling factors in other tissues and organs (Lee et al., 2016). During Hh signal transduction, binding the Hh ligand to patched (PTC), an Hh receptor, stops the suppression of the transmembrane protein, smoothened (SMO), which causes stabilization of the glioma-associated oncogene 2 (GLI2) transcription factor. GLI2 then translocates into the nucleus and induces the expression of Hh target genes such as GLI1 (McMahon, 2000; Riobo & Manning, 2007). SHH, one of three components of the Hh gene family, is expressed in the epithelium of the dental lamina, in an enamel knot during the cap stage, in the inner enamel epithelium and ameloblasts during cell differentiation, and the mesenchymal layer as well (Hardcastle, Hui, & Sharpe, 1999; Zhang et al., 1999). Studies using mice with selectively deleted SHH from the ectoderm have shown that SHH has a significant role in both tooth growth and morphology, mostly through its actions in the earlier stages of tooth development (Dassule, Lewis, Bei, Maas, & McMahon, 2000; Gritli-Linde et al., 2002). Studies on the role of SHH during the development of periodontal tissues, especially its effects in cementoblasts, however, have yet to be reported.

This study is therefore aimed at providing new clues about periodontal tissue regeneration by identifying changes in the expression of various Hh signaling molecules during cementoblast differentiation.

2. Materials and methods

2.1. Cell culture and reagents

The OCCM-30 immortalized cementoblast cell line, which was derived from the dental root surface of transgenic mice containing an SV 40 large T-antigen, was kindly provided by Dr. Martha J. Somerman (National Institutes of Health, Bethesda, MD, USA). The OCCM-30 cells were maintained in a growth medium containing Dulbecco's Minimum Essential Medium (DMEM, Gibco BRL, MD, USA) with 10% fetal bovine serum (FBS, BioWest, Miami, Fla, USA), 100 U/ml penicillin, and 100 μg/ml streptomycin (Gibco BRL). When the cells reached a 70–80% confluence, they were used for subsequent experiments. For cementoblast differentiation, the cells were plated at 5×10^3 cells/cm^2 and allowed to adhere overnight. The medium was then replaced with a differentiation medium containing DMEM with 2% FBS, 50 μg/ml ascorbic acid 2-phosphate, 10 mM β-glycerophosphate (Sigma Aldrich, St Louis, MO, USA), 100 U/ml penicillin, and 100 μg/ml streptomycin in the absence or presence of Hh signaling modulator/BMP7. Differentiation media were changed every other day. Alkaline phosphatase assay on 3 day and alizarin red S staining on 6 day were performed, respectively.

Hh signal modulators, Cyclopamine (Cyclo) and Purmorphamine (Purmo) were purchased from Sigma-Aldrich (ST Louis, MO, USA) and recombinant mouse BMP7 from R&D Systems (Minneapolis, MN, USA).

2.2. Reverse transcriptase-polymerase chain reaction (RT–PCR)

The total RNA was extracted using Trizol Reagent (Invitrogen, Carlsbad, CA, USA). Three micrograms of the total RNA and oligo dT_{18} were then incubated at 80 °C for 5 min. The cDNA synthesis reaction was performed at 42 °C for 1 h with Moloney Murine Leukemia Virus (Promega, Madison, WI, USA) reverse transcriptase, followed by incubation at 94 °C for 5 min to inactivate the reverse transcriptase. For the PCR reaction, AccPower PCR PreMix (Bioneer, Daejeon, Korea) was